# Mode of Action of Streptolydigin

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Streptolydigin and rifamycin inhibit the catalytic function of ribonucleic acid (RNA) polymerase. Streptolydigin can inhibit polymerization after the reaction has started, whereas rifamycin is effective only if it is preincubated with RNA polymerase prior to the addition of substrates. The same relationships are observed with respect to these two antibiotics if the nucleoside triphosphate-pyrophosphate exchange reaction is used in the assay system. The inhibitory effect of streptolydigin is reversible by further addition of RNA polymerase but not by addition of deoxyribonucleic acid to the assay system.

Streptolydigin is an antibiotic produced by *Streptomyces lydigus*. Isolation, characterization, and biological properties have been reported (3, 4, 6), and the structure has been determined (7; Fig. 1).

A more complete bibliography as well as compilation of physical and chemical properties of this drug have been prepared by Umezawa et al. (9).

As part of a study of antibiotics which affect protein synthesis, we observed that streptolydigin could inhibit amino acid incorporation into protein by intact cells of *Bacillus megaterium*. The inhibitory effects on uracil incorporation, however, occurred with a shorter lag, which suggested a more immediate effect on RNA synthesis. A comparison of streptolydigin with rifamycin, an antibiotic inhibitor of ribonucleic acid polymerase, revealed certain similarities and differences with respect to the nature of the inhibition produced by these two antibiotics.

## MATERIALS AND METHODS

**Incorporation by intact cells of B. megaterium.** *B. megaterium* strain B-5 was obtained from H. Halvorson. This strain was cultivated in Antibiotic Medium 3 (Difco), 17.5 g per liter. Isotopic incorporation studies were performed by addition of $^{14}$C-tyrosine (specific activity, 300 $\mu c/\mu$mole), 2 $\mu c$; $^{14}$C-uracil (specific activity, 30 $\mu c/\mu$mole), 2 $\mu c$; or $^3$H-thymidine (specific activity, 1,900 $\mu c/\mu$mole), 2 $\mu c$, to growth flasks containing 10 ml of medium, which were inoculated with 1 ml of an overnight culture. The growth flasks were then agitated in a rotary action shaker-incubator at 37 C, and, at appropriate times, 1-ml samples were withdrawn, filtered (Millipore Corp., Bedford, Mass.; HAWP-02500), and washed with 10 ml of fresh growth medium. The membrane filters were dried and counted in a gas-flow counter at 20% efficiency for $^{14}$C and at 40%

efficiency for $^{32}$P, or in a liquid scintillation counter at 10% efficiency for $^3$H.

Streptolydigin (batch 2923-DEV-22) was developed by the Upjohn Co., Kalamazoo, Mich., and rifamycin SV is a product of Lepetit, Milan, Italy.

**RNA polymerase assay.** The RNA polymerase assay was performed by the method of Chamberlin and Berg (1). The reaction mixture (100 $\mu$liters) contained tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 8.0), 0.04 M; denatured calf thymus deoxyribonucleic acid (DNA), 35 $\mu$g; *Escherichia coli* RNA polymerase (specific activity, 28,000 units/mg), corresponding to fraction IV of Chamberlin and Berg (1), 16 $\mu$g; $^{12}$C-adenosine triphosphate, -cytidine triphosphate (CTP), and -uridine triphosphate (UTP); $^{14}$C-guanosine triphosphate (GTP; specific activity 1 $\mu c/\mu$mole) 0.004 M each, and antibiotic as indicated. After incubation for 20 min at 37 C, the reaction was terminated by addition of 100 $\mu$liters of 10% trichloroacetic acid; the mixture was filtered and counted.

**Pyrophosphate exchange assay.** Pyrophosphate exchange was assayed by a method similar to that employed by Goldberg et al. (5). The reaction mixture (total volume, 150 $\mu$liters) contained: Tris-hydrochloride (*p*H 7.9), 0.1 M; MgCl$_2$, 0.1 M; GTP, CTP, and UTP, 0.004 M each; sodium pyrophosphate-$^{32}$P (specific activity, 2,000 $\mu c/\mu$mole), 0.1 mM; *E. coli* RNA polymerase (specific activity, 28,000), corresponding to fraction IV of Chamberlin and Berg (1), 16 $\mu$g; and antibiotic as indicated. The reaction was terminated by addition of 1 mg of bovine serum albumin and 250 $\mu$liters of 10% trichloroacetic acid to the reaction mixture. Precipitated proteins were removed by centrifugation, and the $^{32}$P-labeled nucleotides were adsorbed on acid-washed charcoal, 100 $\mu$liters of a 15% (w/v) suspension in 0.01 M Na$_4$P$_2$O$_7$. The charcoal suspension was washed three times with 1.5-ml portions of (sodium) acetate buffer, 0.1 M (*p*H 4.5), containing 0.01 M Na$_4$P$_2$O$_7$. The final suspension was collected by filtration through Whatman GF-A glass fiber filters. The filters

were dried, glued to aluminum planchets with the charcoal side facing the aluminum, and counted.

## RESULTS

The effect of streptolydigin on $^3$H-thymidine, $^{14}$C-uracil, and $^{14}$C-tyrosine incorporation by intact cells of *B. megaterium* was first tested. Upon addition of 50 μg of streptolydigin per ml (0.07 mM) to the growth medium, $^3$H-thymidine incorporation continued at its original rate for at least 10 min, $^{14}$C-uracil incorporation stops within less than 5 min, and $^{14}$C-tyrosine incorporation proceeds at its former rate for about 5 min and then stops (Fig. 2). We inferred from these data that the primary inhibitory effect of streptolydigin was on uracil utilization, presumably RNA synthesis, and that the lag in inhibition of tyrosine incorporation could be attributed to utilization of

messenger RNA formed prior to streptolydigin addition. We next attempted to characterize the dose-response and time-dependence of streptolydigin action in a cell-free system. In these studies, a partially purified RNA polymerase from *E. coli* was used. The dose-response of $^{14}$C-GTP polymerization to increasing levels of streptolydigin is shown in Fig. 3. Rifamycin, a known inhibitor of RNA polymerase (8, 10, 11), was included for comparison. From these data, we conclude that streptolydigin can inhibit RNA synthesis in vitro and that rifamycin is about 100-fold more effective.

For comparing the time-dependence of the inhibitory effects of these antibiotics, the lowest concentration capable of completely inhibiting $^{14}$C-GTP incorporation was used. The inhibitory effect of streptolydigin on polymerization is seen
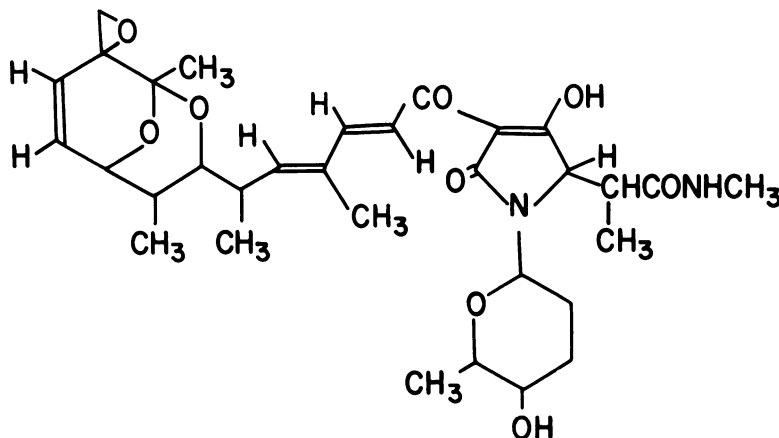
## STREPTOLYDIGIN
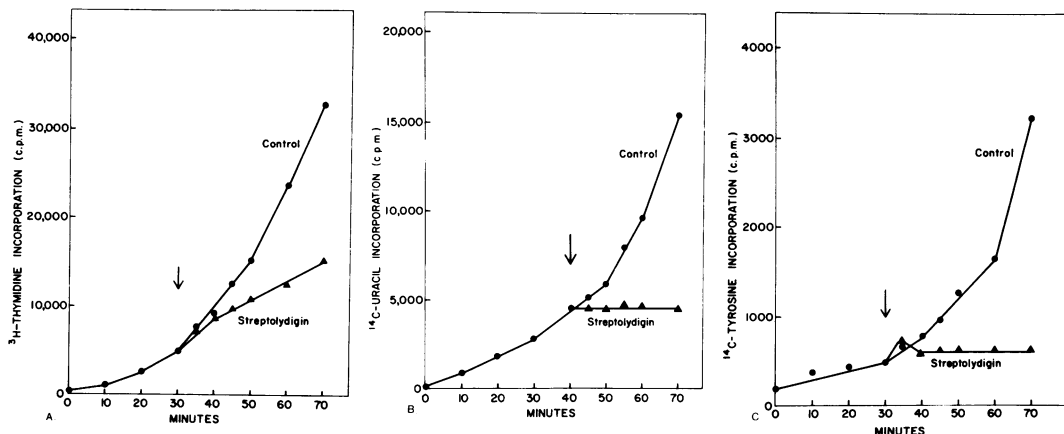


Fig. 1. *Structure of streptolydigin.*



Fig. 2. *Effects of streptolydigin on incorporation of (A) $^3$H-thymidine, (B) $^{14}$C-uracil, and (C) $^{14}$C-tyrosine by intact cells of B. megaterium. Time of addition of streptolydigin is indicated by the arrow.*

within less than 3 min, whereas, in the presence of rifamycin, polymerization continues at its former rate for at least 6 min (Fig. 4). The latter observation is in agreement with the findings of



FIG. 3. *Effects of streptolydigin and rifamycin on incorporation of $^{14}$C-GTP in vitro into acid-insoluble product, dose response. Under the conditions used in the assay, 100% corresponds to 384 cpm or to 7.2 pmoles of GTP incorporated.*



FIG. 4. *Effects of streptolydigin and rifamycin on incorporation of $^{14}$C-GTP, in vitro, into acid-insoluble product, time course. Under the conditions used in the assay, 1,000 cpm corresponds to 19 pmoles of GTP incorporated. Rifamycin (1.4 $\times$ 10$^{-6}$ M) or streptolydigin (1.4 $\times$ 10$^{-4}$ M) were added at 9 min as indicated.*

Umezawa et al. (10) and of Sippel and Hartmann (8), who observed that rifamycin was ineffective as an inhibitor of RNA synthesis if added after the reaction had begun.

RNA polymerase can catalyze the exchange of pyrophosphate with the $\beta$ and $\gamma$ positions of ribonucleoside triphosphates (5). We also compared the inhibitory effects of streptolydigin and rifamycin with respect to the dose-response and time-course of inhibition of this reaction. The results obtained (Fig. 5 and 6) are parallel to those found in the polymerization studies. Pyrophosphate exchange is also approximately 100-fold more sensitive to rifamycin than to streptolydigin. However, upon addition of streptolydigin, the exchange reaction is inhibited within less than 5 min, whereas, in the presence of rifamycin, this reaction proceeds at the control rate for at least 20 min.

To rule out, in a more definitive way, a possible direct effect of streptolydigin on protein synthesis, we attempted to inhibit cell-free protein synthesis directed by poly U, poly UC, poly A, and MS-2 phage RNA as messenger. No inhibition of protein synthesis was observed (Table 1).

The isolation of streptolydigin-resistant enzyme from a resistant mutant would provide evidence in support of the localization of streptolydigin action on RNA polymerase. We have not yet isolated such a mutant. Therefore, to distinguish between inhibition by streptolydigin of the catalytic function of RNA polymerase and inhibition of the template function of DNA, we attempted to reverse the inhibitory effect of strepto-
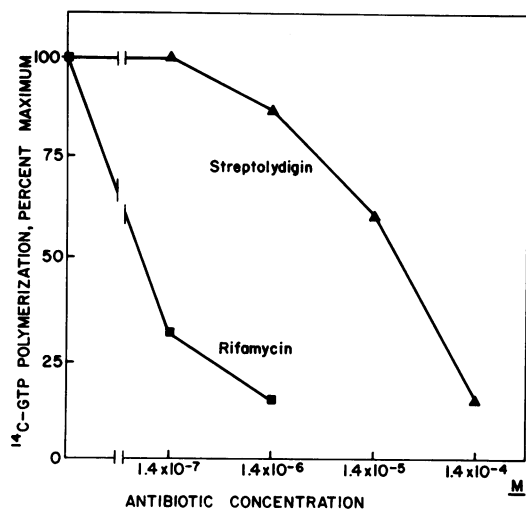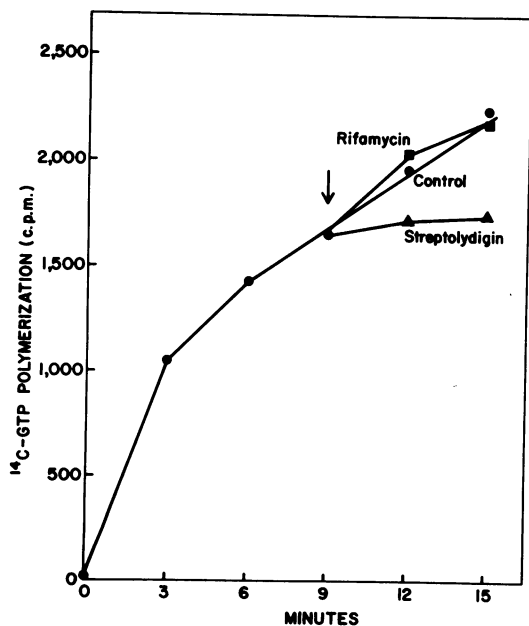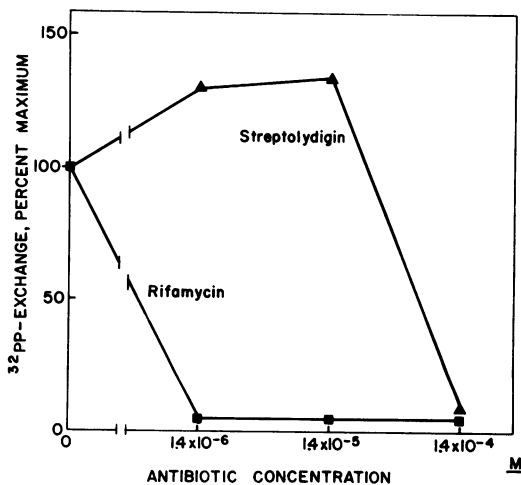


FIG. 5. *Effects of streptolydigin and rifamycin on the $^{32}$P-pyrophosphate-ribonucleoside triphosphate exchange reaction, dose-response. Under the conditions used in the assay, 100% corresponds to 2,349 cpm or to 16 pmoles of pyrophosphate.*
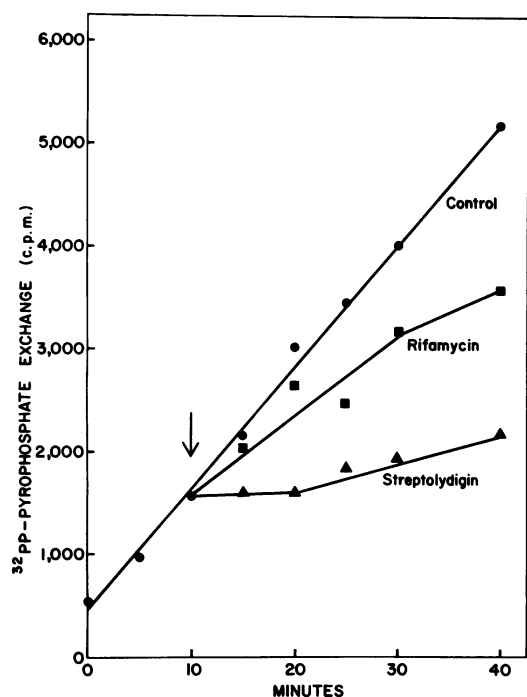
FIG. 6. *Effects of streptolydigin and rifamycin on the $^{32}$P-pyrophosphate-ribonucleoside triphosphate exchange reaction, time course. Under the conditions used in the assay, 1,000 cpm corresponds to 6.6 pmoles of pyrophosphate. Rifamycin (1.4 × 10$^{-6}$ M) or streptolydigin (1.4 × 10$^{-4}$ M) were added at 10 min as indicated.*

TABLE 1. *Effect of streptolydigin on protein synthesis in cell-free extracts from B. stearothermophilus*

| | | Incorporation[a] | | |
|---|---|---|---|---|
| $^{14}$C-amino acid | Messenger RNA | Complete system | Minus template | Complete system plus streptolydigin[b] |
| | | *cpm* | *cpm* | *cpm* |
| Phenylala-nine | Poly U | 27,373 | 1,227 | 31,105 |
| Proline | Poly UC (5:1) | 2,585 | 465 | 2,439 |
| Lysine | Poly A | 11,896 | 561 | 9,729 |
| Alanine | MS-2 phage RNA | 956 | 317 | 885 |

[a] Protein synthesis in cell-free extracts of *B. stearothermophilus* was performed as previously described (2). Reaction mixtures were supplemented with appropriate messenger RNA [poly U, 50 µg/ml; poly UC (5:1), 50 µg/ml; poly A, 100 µg/ml; or MS-2 phage RNA, 100 µg/ml] and streptolydigin as indicated.

[b] Concentration, 2.8 × 10$^{-4}$ M.

lydigin by supplementing an inhibited reaction with RNA polymerase or with DNA. We found that additional RNA polymerase was effective in reversing the inhibitory effect of streptolydigin whereas addition of DNA was not (Table 2).

## DISCUSSION

Streptolydigin and rifamycin inhibit the synthesis of RNA both in intact cells and in cell-free reactions. From the dose-response studies, the sensitivity of *E. coli* RNA polymerase toward these two antibiotics differs by a factor of about 100 with respect to both polymerization and PP-exchange. Because gram-positive organisms are generally more sensitive than gram-negative organisms, they provide a more suitable test system for studying the effects of these antibiotics in intact cells.

When streptolydigin and rifamycin are added to a cell-free polymerization reaction mixture after incubation has begun, different effects are noted. On addition of streptolydigin, both RNA synthesis and pyrophosphate (PP) exchange stop immediately, i.e. within less than 5 min. Whereas on addition of rifamycin, both reactions proceed

TABLE 2. *Reversal of the inhibitory effect of streptolydigin on RNA synthesis in vitro*

| Expt | Incorporation[a] |
|---|---|
| | *cpm* |
| Complete (no inhibitor)......... | 741 |
| Streptolydigin[b].................. | 216 |
| Streptolydigin + 3 × RNA polymerase.................... | 1,210 |
| Streptolydigin + 3 × DNA..... | 325 |

[a] Cell-free polymerization reactions in which *E. coli* RNA polymerase was used were inhibited by streptolydigin, 1.4 × 10$^{-4}$ M. Reaction mixtures otherwise had the composition described. The reaction mixtures were further supplemented with additional RNA polymerase or DNA as indicated. Under conditions of the assay, 741 cpm corresponds to 13.9 pmoles incorporated.

at the same rate for at least 5 min. The relative insensitivity of RNA polymerization to rifamycin once the reaction has started has been interpreted in terms of the inhibition of the initiation reaction (8, 10). The type of experimental design used in arriving at this conclusion, however, does not distinguish between two different classes of mechanisms, one of which indeed involves the inhibition of some reaction which is peculiar to the initiation step and another by which the rifamycin-sensitive site is only accessible when the enzyme is not in contact with its substrate(s). In any case, the difference in sensitivity of RNA polymerization as well as PP-exchange toward

streptolydigin and rifamycin suggests that different mechanisms of inhibitory action are involved; the fact that only additional enzyme was effective in reversing the inhibitory effect of streptolydigin suggests that RNA polymerase and not DNA is the site of action of streptolydigin. One further difference between these antibiotics worth noting concerns the reversibility of their inhibitory effect. In studies on *S. aureus* (data not shown), we have found that inhibition by streptolydigin was easily reversible by removal of the antibiotic, whereas inhibition by rifamycin was not.

In conclusion, streptolydigin inhibits RNA synthesis and it acts on the catalytic function of RNA polymerase. Its mechanism of action differs from that of rifamycin.

## ACKNOWLEDGMENTS

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